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Table of Contents

Page

Introduction…….. 4

Body…….. 4

Key Research Accomplishments…….. 8

Reportable Outcomes…….. 8

Conclusion…….. 8

References…….. 9

Appendices…….. N/A

INTRODUCTION

Substantial evidence supports the view that epigenetic changes play an important role in the development of human prostate cancer (PCa).[1] Identification of these changes will have significant impact on the prevention, diagnosis, prognosis, and treatment of PCa.[2] Induced-pluripotent stem (iPS) cells generated by forced expression of certain transcription factors resemble embryonic stem (ES) cells in their morphology, gene expression, and ability to differentiate into any cell type,[3] therefore, promise nearly everything that ES cells do, including the potential for cell therapy, drug screening and disease modeling.[4, 5] Because iPS cells re-establish a genome-wide epigenetic pattern characteristic of ES cells, iPS cells derived from primary PCa (PCa-iPS) cells can be a powerful tool to identify epigenetic changes responsible for PCa development. We hypothesize that primary PCa cells can be reprogrammed to a pluripotent state by introducing a defined and limited set of transcription factors and by culturing under ES cell conditions. Furthermore, these PCa-iPS cells can be re-differentiated back to PCa cells similar to those in the primary cancer by culturing under differentiation-inducing conditions. By comparing the epigenetic state of PCa-iPS cells and their differentiated progeny, alterations responsible for the cancer phenotype that are erased during the reprogramming can be identified on a genome-wide scale. Our specific aims are: 1) generating PCa-iPS cells using cultured primary epithelial cells derived from PCa (E-CA) by retroviral infection of E-CA cells with viruses carrying Oct4, Sox2, Klf4, and c-Myc, 2) determining cellular and molecular characteristics of PCa-iPS including long-term proliferation potential, embryonic stem cell marker expression, and DNA methylation status within the promoter region of pluripotency genes as well as the potential to differentiate into lineages representative of the three embryonic germ layers and the three cell types (basal epithelial, secretory epithelial, and neuroendocrine) that encompass the prostate epithelium as well as cancer cells that resemble the parental primary PCa cells, 3) dissecting epigenetic changes during PCa-iPS re-differentiation by mapping global DNA methylation during PCa-iPS re-differentiation and identifying genomic sites occupied by PcG proteins in PCa-iPS cells and their differentiated progeny.

BODY

Our first designated task was to generate PCa-iPS cells using E-CA cells (month 1-12). Our specific goals were to **(a)** Retroviral infect E-CA cells with lentiviruses expressing Oct4, Sox2, Klf4, and c-Myc and **(b)** pick and expand iPS cell colonies. We have accomplished (a) and (b) of this aim.

(a) We obtained lentiviral vectors (Oct4, Sox2, Kif4, and c-Myc) and 293T cells from our co-investigator Dr. Wu's lab. 293T cells were plated at ~80% confluency per 100-mm dish and transfected with 12 μg of each lentiviral vector plus 8 μg packaging plasmids and 4 μg VSGV plasmids using Lipofectamine 2000 following the manufacturer's instructions. Medium was changed 16 hrs posttransfection. Viral supernatant was collected 40 hr and 64 hr posttransfection, filtered through a 0.45 μm pore-size cellulose acetate filter, and mixed with PEG-it Concentration Solution overnight at 4°C. Viruses were precipitated at 1,500Xg the next day and resuspended in Opti-MEM medium. E-CA cells were cultured in complete MCDB 105 medium at 37°C in a 5% CO_2 incubator and passage 3 cells were used for reprogramming. E-CA cells ($1X10^5$) were first transduced with individual lentiviruses containing Oct4, Sox2, Klf4, and c-

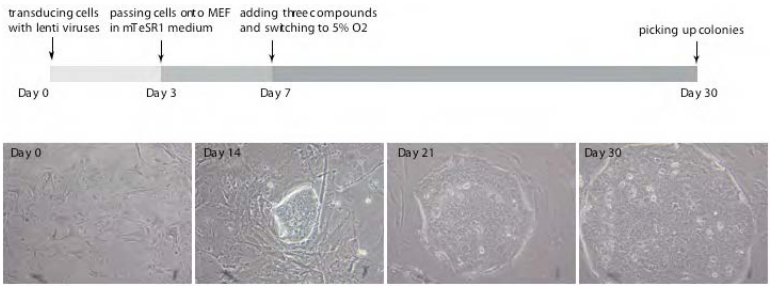

Figure 1 Generation of iPS cells from primary epithelial cells derived from PCa. (Top) Schematic diagram of reprogramming procedures and (Bottom) Images of primary PCa cells at day 0 and putative PCa-iPS colonies from day 14 to day 30. Magnification is 20X.

Myc at a 1:1:1:1 ratio on day 0 in complete MCDB 105 medium. On day 3, cells were transferred onto mouse embryonic fibroblast (MEF) feeder layers with the culture medium switched to human ES cell growth medium mTeSR-1. On day 7, fresh mTeSR-1 medium with 2 μM SB431542, 0.5 μM PD0325901, and 0.5 μM Thiazovivin was added to the cells and cells were incubated in a 5% O_2 chamber. Previous studies have shown that these three compounds and low oxygen enhance the efficiency of reprogramming.[6, 7]

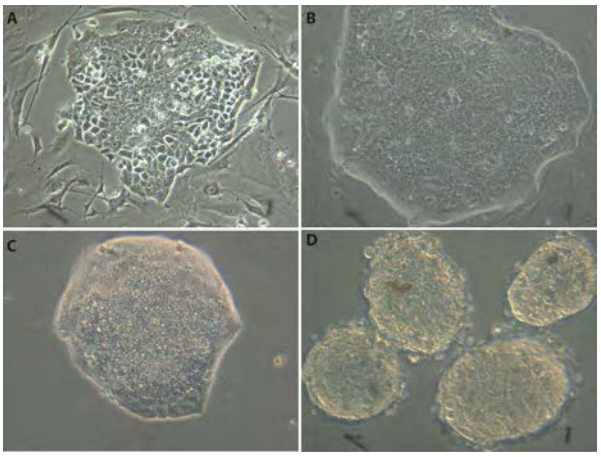

Figure 2 Morphology of putative PCa-IPS cells under different culture conditions (A) on MEF in mTeSR, (B) on MEF in DMEM/F12 (1:1), 10 ng/ml bFGF, 10 ng/ml LIF, and 5% FBS, (C) on Matrigel coated plate in DMEM/F12 (1:1), 10 ng/ml bFGF, 10 ng/ml LIF, and 5% FBS, (D) on collagen coated plate in DMEM/F12 (1:1), 10 ng/ml bFGF, 10 ng/ml LIF, and 5% FBS Magnification for (A), (B) and (D) is 20X, for (C) is 10X

We reprogrammed E-CA cells derived from three different primary carcinomas and obtained 1-7 colonies from each E-CA culture. Figure 1 shows the schematic diagram of the reprogramming procedure and images of putative PCa-iPS colonies over time. These putative PCa-iPS colonies showed typical characteristics of hES cells including defined boundaries and high nuclear-to-cytoplasmic ratio. We mechanically isolated these colonies between day 21-30 and transferred them onto MEF for further expansion. However, we noticed that when maintained in mTeSR-1 medium, these colonies started to become heterogeneous in that large cells with low nuclear-to-cytoplasmic ratio appeared in the tightly packed colonies containing small cells with high nuclear-to-cytoplasmic ratio (Figure 2A). To optimize the maintenance of putative PCa-iPS cells, we tested different media and found that medium containing DMEM/F12 (1:1), 10 ng/ml bFGF, 10 ng/ml LIF, and 5% FBS worked the best for maintaining the morphology of putative PC-iPS colonies (Figure 2B). More importantly, putative PCa-iPS cells formed ES-like colonies without MEF feeder layer in this medium on Matrigel-coated plates (Figure 2C). Finally, putative PCa-iPS cells formed spheres in this medium when grown on other substrates such as collagen and gelatin (Figure 2D) or in ultralow attachment plates. We further expanded the putative PCa-iPS cells on Matrigel-coated plates in DMEM/F12 (1:1), 10 ng/ml bFGF, 10 ng/ml LIF, and 5% FBS, froze down cells in 90% FBS plus 10% DMSO, and stored in liquid nitrogen.

Our second designated task was to characterize and re-differentiate PCa-iPS cells (month 6-24). Our specific goals were to: **(**a) determine cellular and molecular characteristics of PCa-iPS including long-term proliferation potential, embryonic stem cell marker expression, and DNA methylation status within the promoter region of pluripotency genes in reprogrammed cells with comparison of these characteristics to ES cells and parental prostate cancer cells, (b) assess the potential of PCa-iPS cells to differentiate into lineages representative of the three

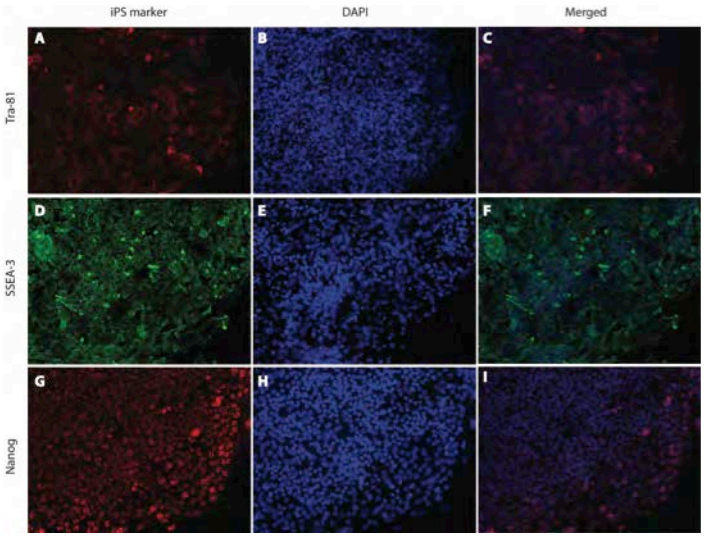

Figure 3 Immunofluoresence staining of putative PCa-IPS cells using antibodies against iPS cell markers Cells were grown on MEF in DMEM/F12 (1:1), 10 ng/ml bFGF, 10 ng/ml LIF, and 5% FBS Nuclear staining of Tra-81 was shown in (A) and (C), SSEA3 in (D) and (F), and Nanog in (G) and (I) DAPI staining was shown in (B), (E) and (H) for the same colonies Magnification is 20X

embryonic germ layers, and (c) evaluate expression of prostate stem cell markers in PCa-iPS cells and their ability to differentiate into the three cell types (basal epithelial, secretory epithelial, and neuroendocrine) that encompass the prostate epithelium as well as cancer cells that resemble the parental primary PCa cells. We achieved most of the elements in (a), all elements of (b), and some elements of (c).

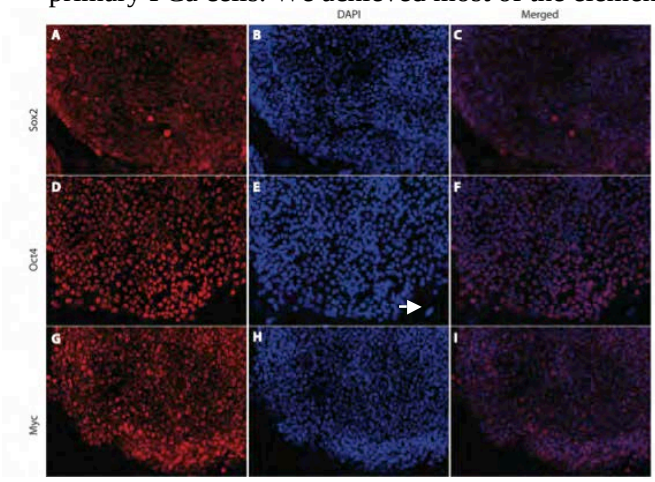

Figure 4 Immunofluoresence staining of putative PCa-iPS cells using antibodies against reprogramming factors Cells were grown on MEF in DMEM/F12 (1:1), 10 ng/ml bFGF, 10 ng/ml LIF, and 5% FBS Nuclear staining of Sox2 was shown in (A) and (C), Oct4 in (D) and (F), and Myc in (G) and (I) DAPI staining was shown in (B), (E) and (H) for the same colonies Magnification is 20X

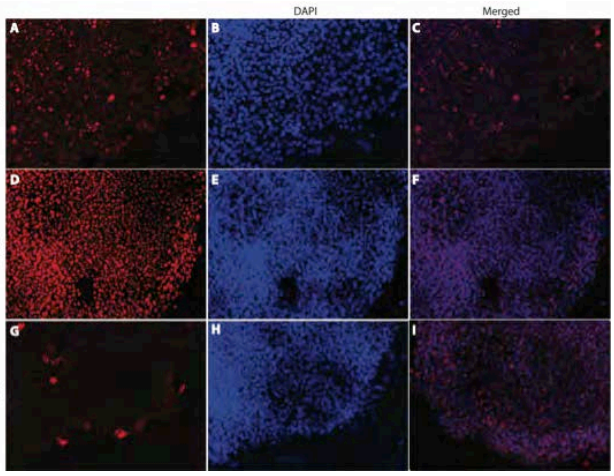

Figure 5 Immunofluoresence staining of putative PCa-IPS cells using antibodies against other markers Cells were grown on MEF in DMEM/F12 (1:1), 10 ng/ml bFGF, 10 ng/ml LIF, and 5% FBS Nuclear staining of Ki67 was shown in (A) and (C), Ku70 in (D) and (F), and cytoplasmic staining for CK5 in (G) and (I) DAPI staining was shown in (B), (E) and (H) for the same colonies Magnification is 20X

We first characterized the immunophenotype of the putative PCa-IPS cells by immunofluorescence staining. As shown in Figure 3, colonies grown on MEF DMEM/F12 (1:1), 10 ng/ml bFGF, 10 ng/ml LIF, and 5% FBS expressed multiple well-known iPS cell markers including cell surface marker Tra-81 (Figure 3A and 3C) and SSEA-3 (Figure 3D and 3F) as well as nuclear marker Nanog (Figure 3G and 3I). Colonies grown on Matrigel in the same medium showed similar expression patterns of the markers (data not shown). In contrast, the parental E-CA cells didn't express any of these markers. In addition, putative PCa-iPS cells displayed nuclear expression of Sox2 (Figure 4A and 4C), Oct4 (Figure 4D and 4F), and c-Myc (Figure 4G and 4I), although it is not clear whether the expression was from endogenous genes or the lentiviral vectors. Note that an MEF nucleus stained with DAPI (arrow in Figure 4E) was negative for Oct4 (Figure 4D). Moreover, putative PCa-iPS cells expressed high levels of Ki67 in the nucleus (Figure 5A and 5C), suggesting that these cells are highly proliferative. To confirm the human origin of these cells, we stained PCa-iPS cells with antibody against human specific nuclear antigen Ku70. PCa-iPS cells were positive for Ku70 (Figure 5D and 5F) whereas MEF cells were negative. Finally, we determined whether the occasional large cells with small nuclear-to-cytoplasm ratio were cytokeratin positive. We found that these cells expressed cytokeratin 5 in the cytoplasm (Figure 5G and 5I) but not cytokeratin 18 (data not shown).

We then compared expression levels of pluripotency genes and reprogramming factors in putative PCa-iPS cells by qPCR. As shown in Figure 6, Klf4 and c-Myc showed much higher expression in PCa-iPS cells compared to ES cells. In addition, the prostate stem cell marker CD133 was expressed more than 10-fold higher in PCa-iPS compared to ES cells (Figure 6). On the other hand, Oct4 expression was ~30% of that in ES cells, and Sox2 and Nanog levels were negligible compared to ES cells (Figure 6). These results suggest that the expression profile of PCa-iPS cells is different from that of ES cells. We also determined how much of the expression of Klf4, c-Myc, and Oct4 was from endogenous copy of the genes rather than from lentiviral vectors by measuring the total level of expression and the expression level from the endogenous genes (Figure 6). Expression of Oct4 was ~100% from the endogenous copy of the gene, suggesting the lentiviral gene was silenced after initial reprogramming. About 13% of Klf4

expression was from the endogenous copy of the gene and almost all Myc expression was from the lentiviral vector. These results suggest that reprogramming factors were differentially expressed and later silenced in putative PCa-iPS cells.

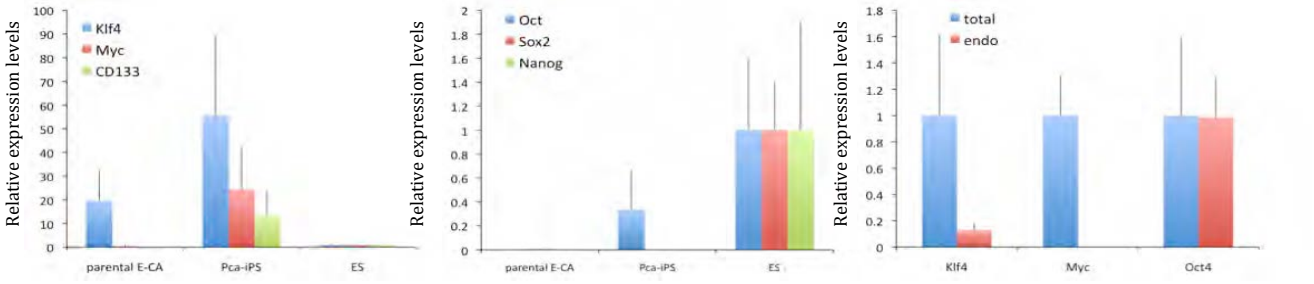

Figure 6 Comparing gene expression levels in E-CA, PCa-IPS and ES cells by qPCR. Expression levels were normalized against those in ES cells. Each reaction was done in triplicate. Error bars represent standard deviation.

Next, we determined whether the putative PCa-iPS cells can differentiate into derivatives of three embryonic germ layers using a teratoma assay. One million PCa-iPS cells were collected from Matrigel-coated plates and resuspended in DMEM/F12 and Matrigel (1:1). Cells were injected either subcutanously in the dorsal flank or under the renal capsule of RAG2$^{-/-}\gamma C^{-/-}$ mice. After 4-6 weeks, tumors were harvested, and fixed with 10% formalin. Paraffin-embedded tissue sections were stained with hemotoxylin and eosin. The histology of the tissues was similar regardless the site where they were implanted. Specifically, the putative PCa-iPS cells formed poorly differentiated carcinomas both under the skin (Figure 7A) and the renal capsule (Figure 7B). However, the tumors were much bigger when grown under the renal capsule compared to the subcutaneous site. Immunohistochemistry using antibody against Ku70 confirmed the human origin of the tumor cells (Figure 7C). These tumor cells didn't express prostate specific antigen (PSA) (Figure 7D), suggesting that they are not differentiated into prostate cancer cells. To determine whether putative PCa-iPS cells can differentiate into cells of prostate lineage, we combined these cells with urogenital sinus mesenchyme (UGM) and supplemented the mice with testosterone, two conditions that promote prostate differentiation.[8] However, the histology of the tumors formed under the renal capsule was similar to those without UGM and testosterone, and they were negative for PSA (data not shown).

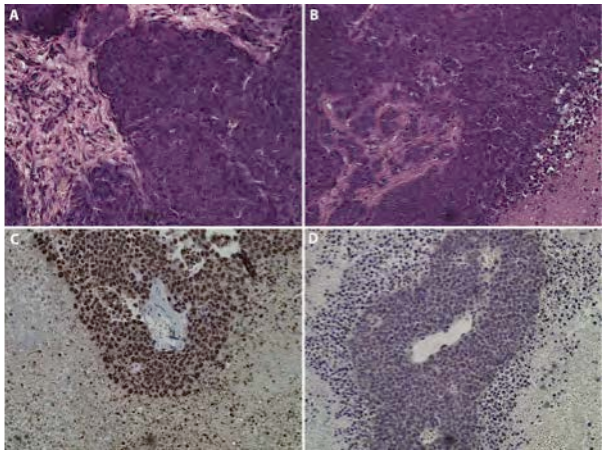

Figure 7 Images of poorly differentiated carcinomas formed in immunodeficient mice by putative PCa-iPS cells (A) H&E staining of tumor cells harvested subcutaneously, (B) H&E staining of tumor cells harvested from under the renal capsule, (C) Immunohistochemistry staining with antibody against human specific nuclear antigen Ku70 of tumors harvested from under the renal capsule, (D) Immunohistochemistry staining with antibody against prostate specific antigen (PSA) of tumors harvested from under the renal capsule Magnification is 20X

We also generated putative iPS cells from cultured cells derived from normal prostate tissues. These iPS colonies showed similar morphology as ES cells in that they had defined boundaries and contained tightly packed cells with high nuclear-to-cytoplasm ratio. In addition, these cells expressed similar expression levels of pluripotency genes such as Oct4 compared to putative PCa-iPS cells. They also expressed high levels of c-Myc and Klf4. Little expression of Nanog and Sox2 was observed in these cells. The difference between these cells and PCa-iPS cells was that they didn't express CD133. In vivo, these cells formed tumors with similar histology to PCa-iPS cells but with longer time and smaller size, suggesting that they are less aggressive than PCa-iPS

cells.

KEY RESEARCH ACCOMPLISHMENTS

• Generated putative iPS cells from primary prostate epithelial cells derived from normal and cancer tissues
• Optimized culture conditions to maintain the morphology of putative iPS colonies
• Determined the expression levels of pluripotent genes in putative iPS cells by immunoflurescence staining
• Compared the expression levels of pluripotent genes in putative iPS cells to ES cells by qPCR
• Determined the in vivo differentiation potential of putative iPS cells

REPORTABLE OUTCOMES

None.

CONCLUSIONS

We achieved all elements of our first aim and most of the elements of our second aim. For Aim 1, we met our goals of retroviral infection of E-CA cells with lentiviruses expressing Oct4, Sox2, Klf4, and c-Myc. We developed a protocol to generate iPS colonies that are morphologically identical to ES colonies and optimized growth conditions to maintain the ES-like morphology of iPS cells. We successfully expanded and stored iPS cell colonies for future investigations. For aim 2, we determined the expression levels of pluripotent genes in putative iPS cells by immunoflurescence staining. In addition, we compared the expression levels of pluripotent genes in putative iPS cells to ES cells by qPCR. Moreover, we determined the in vivo differentiation potential of putative iPS cells by teratoma assay.

From our studies so far, we found that putative iPS cells derived from primary prostate epithelial cells can generate ES-like colonies in a feeder-free manner. They also expressed a subset of pluripotency genes at a much higher level compared to ES cells, while displaying little expression for other pluripotency genes. In addition, the lentiviral expression of reprogramming factors was differentially regulated in these cells in that some factors were silenced while others weren't. Finally, these cells formed poorly differentiated carcinomas, which didn't express prostate cell marker PSA, when implanted in immunodeficient mice regardless the level of testosterone or the site of implantation.

Our results suggest that the putative PCa-iPS cells are not completely reprogrammed for two reasons. First, they don't express comparable levels to ES cells of pluripotency genes such as Nanog. Secondly, they don't differentiate into derivatives of the three embryonic layers of cells as do ES cells. Even though they showed similar morphology to ES cells and express some genes typically expressed by ES cells and fully reprogrammed iPS cells, it seems that additional events are needed to further promote reprogramming these cells to the pluripotent state. We will attempt the following modifications to achieve this goal : 1) Introduce six reprogramming factors including Sox2, Oct4, Myc, Klf4, Nanog, and Lin28, 2) Knock down transcription factors that restrict cell lineages such as Sox 9 and Pax 5 by RNAi, 3) Test compounds that inhibit histone deacetylation such as valproic acid to enhance reprogramming of E-CA cells, 4) Use single cells isolated from fresh tissue samples rather than cultured primary cells for reprogramming. Our primary cultures are basal epithelial cell-like in nature, which may behave differently from secretory cells in their capability of being reprogrammed. Another direction of future study is to test conditions that are suitable for differentiation of putative iPS cells in vitro. We are currently determining the identity of cells in the spheres formed by putative iPS cells grown in ultralow attachment plates. Different culture conditions will be tested on these spheres and the cell compositions of

these spheres will be determined by staining with lineage specific markers for secretory and basal epithelial cells.

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APPENDICES

None.

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